Inhibition of apoptosis by 60% oxygen: a novel pathway contributing to lung injury in neonatal rats

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The major pathological features in lungs from infants with BPD in the current era are large simplified distal air sacs and interstitial thickening (17). These changes have been replicated in a number of animal models of BPD, including rats (22), mice (60), lambs (4), and baboons (16). From correlating the pathological findings in human BPD with studies in such animal models has come an appreciation that the simplified distal air sacs observed in human BPD reflect an inhibition or arrest of normal postnatal alveolar formation (17). Formation of alveoli from their large simple precursors, saccules, is largely (85%) a postnatal event in humans born at full term and a completely postnatal event in rats and mice (14). The inhibition or arrest of alveolarization observed in BPD is likely to be mediated by a dysregulation of growth factors that are essential for normal alveolar formation, such as PDGF- AA (39) and -BB (13), VEGF (27), hepatocyte growth factor (HGF; 49), FGF-7 (50), and bombesin-like peptides (54). Recent observations suggest that a failure of alveolar formation can persist into late childhood (18) and that emphysema is present in their late teens in a large percentage of those surviving moderate-to-severe BPD (61).

The factors that contribute to the increased cell mass with interstitial thickening observed in BPD, and animal models of BPD, are unknown. A previously unexplored explanation for these findings is that the interstitial thickening observed in evolving chronic neonatal lung injury can be attributed, at least in part, to an inhibition of physiological lung cell apoptosis. Apoptosis is an integral part of alveolar formation, serving to remove excess cells to optimize gas exchange (52, 53). Distinct signaling pathways that lead to apoptosis have been delineated, and numerous proteins have been identified as either inducers or inhibitors of the apoptotic process. Intrinsic and extrinsic pathways of apoptosis both lead to the cleavage of inactive procaspases into active caspases (31). The intrinsic apoptosis pathway is mediated through the mitochondria and is regulated by members of the Bcl-2 family. These include both proapoptotic proteins (e.g., Bax, Bcl-xS, Bak, and Bad) and antiapoptotic proteins (e.g., Bcl-2, Bcl-xL, and Bcl-w). The Bcl-2 family determines cell death and survival by controlling mitochondrial membrane ion permeability, cytochrome c release, and the subsequent activation of caspase-9 (1, 5). Caspase-9, one of the initiator caspases, subsequently activates effector caspases, including caspases-3, -6, and -7, which trigger the downstream proteolysis, DNA fragmentation and degradation that result in cell death (20).
That inhibition of physiological postnatal apoptosis may contribute to tissue thickening in neonatal lung injury was suggested by a comparison of histological changes following two different interventions. In newborn rats, injection of a truncated soluble FGF receptor-1 (sIIIc) (sFGF-R1) on day 3 of life inhibited normal postnatal lung cell apoptosis when studied at day 7 (63) and resulted in marked parenchymal thickening. Exposing pups to 60% O₂ for 14 days, to cause lung injury (13, 22, 32, 42, 43, 49, 62), resulted in a histology characterized by areas with a marked degree of interstitial thickening and minimal DNA synthesis, mixed with other areas without interstitial thickening and minimal DNA synthesis (22), but with an average increase in the tissue fraction of ~38% (32, 42, 43, 62) by day 14. These observations led us to test the hypothesis that inhibition of apoptosis contributes to the interstitial thickening of the lung observed in the neonatal rat exposed to 60% O₂.

Numerous investigators have examined the effects of very high concentrations of O₂, usually 95–100%, on rodent lungs and observed an increase in lung cell apoptosis (11, 19, 25). We are unaware of other studies that have addressed the relationship between lung cell apoptosis and more moderate O₂ concentrations.

**MATERIALS AND METHODS**

*In vivo procedures.* All animal experiments were conducted in accordance with Canadian Council on Animal Care guidelines. Approvals for animal use were obtained from the Animal Care Review Committees of the Sunnybrook and Hospital for Sick Children Research Institutes. Pathogen-free timed-pregnant Sprague-Dawley rats (250–275 g) were obtained from Taconic (Germantown, NY). Rat search Institutes. Pathogen-free timed-pregnant Sprague-Dawley rats

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**Tissue fraction**. Measurement of the tissue fraction was as previously described (32, 42, 43, 62, 63). Lungs were perfusion fixed while inflated with air under a constant airway pressure of 20 cm water, for preparation and mounting of 5-μm sections. Lung tissue was studied at arbitrary time points of 4, 7, 10, and 14 days of age to span the 14-day exposure period. Counts were performed by observers masked from group allocation. Ten random images were captured from nonoverlapping fields from each section, with four randomly oriented sections per animal and four animals from different litters per group. A 130-point contiguous counting grid superimposed on each (×200) image was used to calculate the tissue fraction per image, by counting the proportion of grid points that fell on tissue.

**Histocheniistry and immunohistochemistry.** Sections that were prepared as above for morphometric analysis were used for immunohistochemistry. An avidin-biotin-peroxidase complex method (24) was used for immunostaining. Slides were incubated with the primary antibody overnight at 4°C. After incubating with biotin-conjugated secondary antibody for 1 h, the labeled Vectastain ABC system (Vector Laboratories, Burlingame, CA) was used with 3,3-diaminobenzidine (SK 4100, Peroxidase Substrate kit, DAB, Vector Laboratories) as a substrate. Hematoxylin was used as the nuclear counterstain. Slides were mounted in Permount mounting medium. A perox-

idase immunohistochemistry detection kit for apoptosis-specific DNA fragmentation was from Millipore (Billerica, MA). This was an initial screening protocol and was only used on samples from pups exposed to air or 60% O₂ for 4 days, at which time point we had previously demonstrated the presence of physiological apoptosis (63). A rabbit polyclonal antiserum against human cleaved caspase-3 (Cell Signaling Technology, Boston, MA) was used at a 1:300 dilution. A goat polyclonal antibody to murine cleaved caspase-7 (Santa Cruz Bio-
technology, Santa Cruz, CA) was used at a dilution of 1:100. A rabbit polyclonal antibody to human survivin (NOVUS Biologicals, Little-
ton, CO) was used at a 1:100 dilution. Goat anti-rabbit IgG or donkey anti-goat IgG biotin-conjugated secondary antibodies were diluted to 1:200 in blocking solution (Santa Cruz Biotechnology). As a negative control, sections were immunostained after immunoadsorption of antibodies with a fivefold excess, by weight, of the blocking peptide against which the antibody was raised. The negative controls for caspases-3 and -7 immunohistochemistry are shown in Supplemental Fig. S1 (the online version of this article contains supplemental data). Immunostaining for cleaved caspase-3 and -7 and survivin was performed at day 4 of exposure, to match the initial assessment of DNA fragmentation, then at the 3-day intervals used in the tissue fraction assessments to identify time points of qualitative maximum protein expression, which could then be used for quantitative assessment by Western blot.

Once staining was complete, group allocations were unblinded to select time points for Western analyses. Images for publication were selected as representative from several images for each animal.

**Western analyses.** Western blot analyses of lung tissue lysates were conducted as previously described (63). On the basis of immunohis-

tochemistry findings for cleaved caspases-3 and -7, and for survivin, Western analyses were limited to tissues from pups exposed to air or 60% O₂ for 4 or 7 days, when the greatest differences between groups might be expected. Supernatants of lung tissue lysates, representing the cytoplasmic fractions, were stored at ~80°C. For analysis of cytoplasmic cytochrome c, samples were centrifuged at 21,000 rpm at 4°C for 30 min. Protein content of extracts was determined using a protein assay kit (Bio-Rad Laboratories, Hercules, CA). Membranes were incubated overnight at 4°C with 1:1,000 dilutions of rabbit polyclonal antibodies to cleaved caspase-3 and -7 and Bax (Cell

**Fig. 1. Density of apoptotic lung cells (A), as detected by TUNEL immunoo-

histochemistry, and cleaved caspase-3-positive cells (B), following exposure of neonatal rat pups to air or 60% O₂ for 4 days. Data are means ± SE and represent values/mm² of the field of view; n = 4 pups, each from a different litter. *P < 0.05 compared with values for air-exposed pups.**
Signaling Technology), 1:500 dilutions for survivin and phosphorylated p38 (p-p38), 1:200 dilutions of mouse monoclonal antibodies to rat cytochrome c and Bcl-xL, or a 1:500 dilution of a goat polyclonal antibody to phosphorylated Erk (p-Erk) (Santa Cruz Biotechnology). Rabbit anti-human FGF-2 or FGF receptor-1 (FGF-R1) antibodies (Santa Cruz Biotechnology) were in 1:200 and 1:500 dilutions, respectively. As an internal control, rabbit anti-α-catenin was used at a 1:4,000 dilution (Sigma, Oakville, ON). After washing, the membranes were incubated with secondary horseradish peroxidase-conjugated anti-mouse antibody (Calbiochem-Novabiochem, San Diego, CA), in 1:10,000 dilution, goat anti-rabbit horseradish peroxidase-conjugated secondary antibody (Cell Signaling Technology), except for p-p38 for which the dilution was 1:2,000, or 1:2,000 donkey anti-goat horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology), for 90 min.

**In vitro studies.** Primary monolayer cultures of mixed lung cells from day 7 rat pups, which contain ~50% type II pneumocytes and 50% mesenchymal cells, were prepared as previously described (56), except that FBS was not hormone depleted with carbon prior to use. Cultures were grown to ~70% confluence, then serum-starved for 24 h prior to studies. DNA synthesis was measured by [3H]thymidine (MP Biomedicals, Irvine, CA) incorporation into cellular DNA (57). Staurosporine-induced apoptotic cell death was assessed by measurement of the release of preincorporated [8-14C]adenine (Sigma) into culture medium (40). Survivin (Abnova, Taipei, Taiwan) was delivered into cells after being complexed with liposomes (Lipofectamine...
2000, Invitrogen) according to the manufacturer’s instructions. A 10-μl aliquot of the stock liposome suspension (1 mg lipid/ml) was added to 2 ml of culture medium that had either no additives or contained 20 or 80 ng/ml survivin. These concentrations were selected on the basis of preliminary dose curves (data not shown). This medium was added to the cells to give a final concentration of 1 μg lipid/cm². For assays of cell death, liposomes were added along with [8-14C]adenine for 24 h. The monolayer was washed prior to the addition of staurosporine (Cayman Chemical, Ann Arbor, MI) to give a final 100 nM concentration for a further 24 h. For studies of DNA synthesis the [3H]thymidine was added for 24 h with the liposomes.

Statistical analyses. A two-tailed Student’s t-test was used to determine significance in all comparisons of paired data. One-way ANOVA, followed by pairwise analysis with Tukey’s test, by using the SigmaStat (SPSS, Chicago, IL) analysis program, was used for comparisons of multiple groups. Values are presented as means ± SE. A P value < 0.05 was regarded as statistically significant.

RESULTS

By qualitative assessment, parenchymal thickening in pups exposed to 60% O₂ was only obvious and reproducible after a 14-day exposure. This was confirmed by a quantitative assessment (Supplemental Fig. S2). Counts of apoptotic day 4 lung cells (Fig. 1A), as identified by immunohistochemistry for apoptosis-specific DNA fragmentation, confirmed the original hypothesis that subsequent parenchymal thickening following
exposure to 60% O₂ was associated with a preceding reduced density of apoptotic lung cells. Further confirmation of this was obtained by counting cleaved caspase-3-positive day 4 lung cells (Fig. 1B), also identified by immunohistochemistry, which gave similar values for cell densities to those obtained for apoptosis-specific DNA fragmentation. These reductions in apoptotic and caspase-3-positive cells/unit area could not be accounted for by a change in tissue mass (Supplemental Fig. S2). Immunohistochemistry for both cleaved caspases-3 (Fig. 2) and -7 (Fig. 3) were used to examine the temporal appearance of apoptotic cells on days 4, 7, 10, and 14 in postnatal rat lungs (63). As previously described (63), cleaved caspase-3 was evident in lung tissue from normal air-exposed pups, with maximal apparent content at days 4 and 7. Exposure to 60% O₂ appeared to reduce cleaved caspase-3 content. A similar pattern was found for cleaved caspase-7 in the lung tissue from rat pups exposed to air, with an apparently reduced content following exposure to 60% O₂. These qualitative observations were further assessed by use of Western blots (Fig. 4A). Cleaved caspases-3 and -7 contents were reduced by exposure to 60% O₂ for 4 days. In contrast, the content of a proapoptotic member of the Bcl-2 family, Bax, was not obviously affected, and the content of the antiapoptotic protein, Bcl-xL, was increased. The proapoptotic release of cytochrome c from mitochondria into lung cell cytoplasm was also reduced by a 4-day exposure to 60% O₂. After normalization to α-catenin, densitometric analysis confirmed (Fig. 4B) statistically significant reductions in the proapoptotic proteins, cleaved caspase-3 and -7, and cytoplasmic cytochrome c in the lungs of day 4 60% O₂-exposed rats, compared with values for animals that had been exposed to air. The antiapoptotic protein, Bcl-xL, was increased in the lungs of animals exposed to 60% O₂ for 4 days. Similar, and statistically significant, effects of 60% O₂ on the contents of cleaved caspases-3, -7, Bcl-xL, and cytoplasmic cytochrome c were also evident on day 7 of life (Fig. 5). When day 4 and day 7 values were compared, there was no significant difference for cleaved caspase-3. Cleaved caspase-7 was significantly reduced and cytoplasmic cytochrome c increased in both air- and 60% O₂-exposed groups at day 7 relative to day 4. Bcl-xL was significantly increased in the air-exposed group and significantly decreased in the 60% O₂-exposed group at day 7 relative to day 4. Because there was no difference in Bax levels between groups at day 4, at which time point there were differences in lung cell apoptosis, we did not pursue changes in Bax at day 7.

On the basis of our previous observation (63), that FGF-2 binding to its receptor FGFR-1α(IIIc) (FRF-R1) regulated physiological apoptosis during normal postnatal rat lung development, we had anticipated that lung contents of FGF-2 and/or the FGF-R1 would be reduced following exposure to

Fig. 4. Analyses of apoptosis-related protein contents in lung tissue from rat pups exposed to air or 60% O₂ for 4 days. A: Western blots showed apparently reduced contents of cleaved caspases-3 and -7, and cytoplasmic cytochrome c, increased content of Bcl-xL, and no change in Bax content following exposure to 60% O₂, compared with animals exposed to air. B: densitometric analyses confirmed these findings. Values for air-exposed tissue shown as open bars and for 60% O₂-exposed tissue as solid bars. Data are means ± SE; n = 4 samples, each from a different litter. Each sample from an individual litter was derived by pooling the lungs of 4 average-sized pups. *P < 0.05 compared with values for air-exposed pups.
60% O2. When assessed by Western blot, there was no effect of 60% O2 on the lung content of either FGF-2 or FGF-R1 at day 4 (Supplemental Fig. S3, A and C) or day 7 (Supplemental Fig. S3, B and C) of life, or for their downstream targets, phosphorylated p38 and phosphorylated Erk-1 at day 4 (Supplemental Fig. S4, A and C) or day 7 (Supplemental Fig. S4, B and C) of life.

Immunohistochemistry was used to screen for the putative antiapoptotic protein survivin, in lung tissues from pups at the day of birth (day 0), and after 4, 7, or 10 days in air or 60% O2 (Fig. 6). Relative to the lung tissue from air-exposed pups, there was an apparent increase in survivin content after 4, 7, and 10 days of exposure to 60% O2. Analysis of Western blots (Fig. 7A) by densitometry (Fig. 7B) confirmed a significant increase in survivin content in lung tissue at days 4 and 7 of exposure to 60% O2, compared with samples from animals exposed to air. Survivin content significantly increased at day 7 relative to day 4 in both air- and 60% O2-exposed groups. The question of whether survivin has antiapoptotic activity in neonatal lung cells was addressed in vitro. Primary monolayer cultures of day 7 mixed lung cells had apoptotic cell death induced by exposure to staurosporine (Fig. 8A). Cells were pretreated with either liposomes alone or liposomes complexed with survivin. Liposomes alone had no effect on cell death. Liposome-associated survivin at 80 ng/ml completely attenuated the staurosporine-induced cell death. Treatment of cells with either liposomes alone, or liposomes complexed with survivin, had no effect on cell DNA synthesis (Fig. 8B). An ~50% increase in DNA synthesis was observed with the 5% FBS positive control.

DISCUSSION

Herein we describe a previously unrecognized contributor to the pulmonary interstitial thickening observed in a neonatal rat model of BPD induced by 60% O2, the inhibition of physiological apoptosis. The developing lung undergoes dramatic tissue growth and remodeling to achieve the mature alveolar architecture required for optimal gas exchange. Prenatally, an effective alveolar-capillary interface is gradually established through a relative thinning of the interstitial compartment by apoptosis (52). Postnatally, apoptosis appears to be involved in trimming off excess type II epithelial cells and fibroblasts to increase lung surface area to enhance gas exchange (53). An inhibition of apoptosis may contribute to the marked relative increase in epithelial cells observed in areas of interstitial thickening in the neonatal rat exposed to 60% O2 (49). An increase in epithelial cells has also been reported in a baboon model of BPD (41).

In this study, as previously reported (63), we observed expression of the proapoptotic proteins, cleaved caspases-3 and...
-7 in normal postnatal rat lung tissue. Caspases-3 and -7 are critical mediators of the mitochondrial events leading to apoptosis, and double-knockout mice lacking caspase-3 and -7 die rapidly after birth (35), which was the rationale for our particular focus on cleaved caspase-3 and -7 as regulators of apoptosis. The peak time points for their expression were at postnatal days 4 and 7, which are within the time frame during which the most rapid increase in alveolar density occurs (45).

Other investigators have reported an increase in lung cell apoptosis following exposure of rats (25) and mice (11, 19) to very high O2 concentrations of 95–100%, which is associated with an arrest of lung growth (30). In contrast, animals that

**Fig. 6.** Immunohistochemistry for the putative antiapoptotic protein, survivin, in lung tissue from pups at the day of birth (day 0), or after 4, 7, or 10 days in air or 60% O2. As a negative control, sections from day 7 60% O2-exposed pups were immunostained after immunoadsorption of the antibody with the blocking peptide (Blocking Peptide) against which the antibody had been raised. Relative to the lung tissue from air-exposed pups, there was an apparent increase in survivin (brown stain) content after 4, 7, and 10 days of exposure to 60% O2. Bar = 100 μm.
were exposed to 60% O_2 had reduced contents of the proapoptotic proteins, cleaved caspase-3 and -7 and cytoplasmic cytochrome c. Our study does not address whether it is the expression of caspases-3 and -7 or their cleavage that is inhibited by 60% O_2. Exposure of neonatal rats to 60% O_2 also resulted in an increased content of the antiapoptotic protein Bcl-xL. The changes in these proteins were not accompanied by any change in Bax at day 4, which is surprising and for which we have no explanation at this time. Increased Bcl-xL, which inhibits cytochrome c release from mitochondria, has also been reported to occur in adult mice exposed to 100% O_2, which was interpreted to be a failed protective response (23). The increase in apoptosis observed in neonatal rat lungs at very high O_2 concentrations (25), and the inhibition of apoptosis observed with exposure to 60% O_2, may simply reflect a concentration-dependent up- or downregulation of apoptosis by reactive O_2 species (15). The accompanying interstitial thickening observed with 60% O_2, but not with very high O_2 concentrations, likely reflects both an inhibition of apoptosis in the former, and an arrested proliferation of those cells whose progeny would otherwise have been destined for apoptosis in the latter, injury.

Survivin is a bifunctional protein widely believed to regulate mitosis and suppress apoptosis (6, 34). It was thought to be expressed in essentially all cancerous, but not normal, adult cells (7). Recent evidence suggests that it may play a role in inhibiting apoptosis in noncancerous lung tissue (64). However, the role of survivin as an inhibitor of apoptosis remains an issue of some debate (38), and we have elected to classify it simply as a putative regulator of apoptosis. Survivin has been reported both to be expressed in lung epithelial cells from the fetal mouse (2, 7) and to play a critical role in monocrotaline-induced pulmonary hypertension in adult rats (44), which led us to test for its presence in the postnatal rat lung exposed to air or 60% O_2. An obvious increase in survivin content was observed in the lungs of 60% O_2-exposed rat pups after 4 and 7 days of exposure. This was unlikely to reflect a mitogenic function because DNA synthesis is reduced after 4 days, and not increased after 7 days, of exposure to 60% O_2 (22), nor did we observe a mitogenic effect in vitro. Support for it playing a role in the observed 60% O_2-mediated inhibition of lung cell apoptosis comes from our in vitro study in which survivin, delivered intracellularly by use of liposomes, inhibited staurosporine-mediated cell death in cultured lung cells. Taken together, we concluded that the 60% O_2-induced increased content of the antiapoptotic protein, Bcl-xL, led to a down-stream inhibition of caspases-3 and -7 activation, with a resultant inhibition of apoptosis. Survivin may also be a contributor to this inhibition.

In tumors, induction of survivin appears to be associated with an increase in the production of growth factors, such as PDGF-BB (12), FGF-2, VEGF (48), and HGF (55), all of which have antiapoptotic properties (33, 46, 51, 59). FGF-7 also has antiapoptotic properties (21). In our model of 60% O_2-induced neonatal lung injury, the lung contents of PDGF-BB (13), FGF-2 (Supplemental Fig. S3), and VEGF (42, 43) are either reduced or unaffected. However, the lung contents of HGF and FGF-7 are increased in distal lung tissue of neonatal rats following exposure to 60% O_2 (49, 58) and could contribute to the observed antiapoptotic effect of 60% O_2. Whether an increase in HGF content following exposure to 60% O_2 is causally related to the increased survivin content is...
not known and will be difficult to assess because inhibition of HGF binding to its receptor arrests lung growth (49). Paradoxically, HGF concentrations in tracheal aspirate samples from premature human infants are inversely correlated with the subsequent risk of developing BPD (36), although whether the source of HGF in these tracheal aspirates is the distal lung or the upper airway is unknown. Lastly, exposure to 60% O₂ is associated with an increase in tissue nitration, likely due to increased macrophage-derived peroxynitrite (29, 32, 42, 43). Peroxynitrite has been reported to inhibit caspase-3 (37) and could therefore also be contributing to the 60% O₂-mediated inhibition of apoptosis. Treating pups exposed to 60% O₂ with a peroxynitrite decomposition catalyst protected against the increase in tissue fraction observed in this model (43).

A limitation of this study is that we have not addressed the questions of which cell type(s) are responsible for the increase in tissue fraction evident at day 14 of exposure to 60% O₂ and how an increase in cell number was achieved. This will require a temporal mapping of lung cell types in parallel with the presence or absence of apoptosis or DNA synthesis in the same cells. There would appear to be a complex interaction between proliferation and apoptosis with respect to tissue fraction, in that decreased cell apoptosis in the first week of exposure to 60% O₂ is not associated with an increase in tissue fraction, presumably because this effect is balanced by the inhibition of DNA synthesis previously reported to occur in this model (22).

A pilot study in premature human infants that correlated FGF-2 and apoptotic cell content of tracheal aspirates on day 1 of life with subsequent death or development of BPD (8). That study reported a correlation between an increase in FGF-2 in tracheal aspirates and both the number of apoptotic cells and the risk of death and/or BPD. Those findings could be perceived as in conflict with our results in neonatal rats but in fact are not. FGF-2 is highly expressed in airway epithelium (30), which would be the major source of FGF-2 in tracheal aspirates. Our studies of FGF-2 expression in neonatal rat lungs were in lung homogenates from which large airways had been specifically removed prior to homogenization. Similarly, the correlation with an increase in apoptotic cells in tracheal aspirates on day 1 of life in human infants is likely to reflect events taking place in the airway rather than in the distal lung. Confirming or rejecting an inhibition of apoptosis, and an increase in survivin content, in human BPD will require the study of autopsy lung tissues samples from preterm infants dying of other causes, such as sepsis or necrotizing enterocolitis, early in the course of their developing BPD.

DISCLOSURES

No conflicts of interest, financial or otherwise are declared by the author(s).

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